

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

STIC-ILL

338,105^W

From: Steadman, David (AU1652)
Sent: Wednesday, March 21, 2001 5:11 PM
To: STIC-ILL
Subject: Literature/ILL Request

2602778

Art Unit: 1652
Room #: 8B-11
Serial #:09/621,448

Please provide the following references:

1) Am J Clin Pathol 1967 Mar;47(3):271-81
Glucose-6-phosphate dehydrogenase, the pentose phosphate cycle, and its place in carbohydrate metabolism.
Horecker BL

2) Folia Microbiol (Praha) 1982;27(6):365-9
Coordinate regulation of the pentose phosphate pathway and of the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (decarboxylating).
Pascual C, Herrera LS

Thank you,
David Steadman

6757069

BEST AVAILABLE COPY

Coordinate Regulation of the Pentose Phosphate Pathway and of the Activity of Glucose-6-phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase (Decarboxylating)

C. PASCUAL and L.S. HERRERA

Department of Microbial Genetics, Centro Nacional de Investigaciones Científicas,
Apartado Postal 6990, La Habana, Cuba

Received December 3, 1981

ABSTRACT. The activities of glucose-6-phosphate dehydrogenase (GPD) and 6-phosphogluconate dehydrogenase (decarboxylating) (PGD) in *Saccharomyces cerevisiae* were found to change by a factor similar to the reported changes in the rate of the pentose phosphate pathway between cells grown in minimal medium and in rich medium. It is suggested that the rate of this pathway is regulated not only by the activity of GPD, a well-known key enzyme, but also by PGD. These two enzymes seem to function in a coordinated fashion.

Pathway and of the	
dehydrogenase (De-	
hydrogenase (De-	365
hydrogenase (De-	370
hydrogenase (De-	377
-α-Amino-ε-capro-	
-hydrogenase (De-	382
-hydrogenase (De-	390
ity and Autolytic	
-hydrogenase (De-	395
-hydrogenase (De-	404
emical Parameters	
-hydrogenase (De-	413
extracts of Bacteria	
-hydrogenase (De-	418
izoplane of Sugar-	
-hydrogenase (De-	423
tions of Inorganic	
-hydrogenase (De-	428
Complex in a Sub-	
-hydrogenase (De-	433
in the Antibiotic-	
-hydrogenase (De-	446
Algae	450
Algae	455
uring Membrane	
-hydrogenase (De-	460

<i>S. subtilis</i> Mutants	
-hydrogenase (De-	465
<i>S. griseus</i> Grown in	
-hydrogenase (De-	468
-hydrogenase (De-	471
-hydrogenase (De-	475

In many biological systems the pentose phosphate pathway plays a key role in the formation of NADPH, pentoses and erythrose necessary for biosynthetic reactions (Pontremoli and Grazi 1969). Due to its properties and position in metabolism, a crucial function in the regulation of the pentose phosphate pathway has been attributed to glucose-6-phosphate dehydrogenase (GPD) (EC 1.1.1.49) (Afolayan 1972; Eggleston and Krebs 1974) while a secondary role has been ascribed to its partner enzyme, 6-phosphogluconate dehydrogenase (decarboxylating) (PGD) (EC 1.1.1.44). Both the induction of enzymes and the modulation of enzyme activity at the substrate level are considered to be important in regulating the capacity of these dehydrogenases (Sapag-Hagar *et al.* 1973; Holten *et al.* 1976).

In *Saccharomyces cerevisiae*, the composition of the medium used for cell growth constitutes an important factor involved in determining the rate of the pentose phosphate pathway. Gancedo and Lagunas (1973) demonstrated that the contribution of the pathway to glucose catabolism is greater in yeast grown in minimal medium than in yeast grown in an enriched medium. Since GPD and PGD have been assigned an important function in regulation it can be expected that the variation in the rate of the pathway observed by Gancedo and Lagunas is due to changes in the activities of the dehydrogenases. Evidence of this prediction as well as a study of several factors involved in the regulation of PGD is reported here.

MATERIALS AND METHODS

Strain. *Saccharomyces cerevisiae* 196-2 (α his6) was obtained from M. Luzzati.

Media. The complete medium (YP) contained (in g/L): yeast extract 10, peptone 10, glucose 20 or 100. The minimal medium was prepared as described by Galzy and

TABLE I. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (decarboxylating) in cells grown in different media

Medium	Substrate	nkat/mg	
		GPD	PGD
Complete	2 % glucose	1.87	2.25
	10 % glucose	2.12	2.57
	10 % glucose ^a	2.93	2.58
	3 % ethanol	4.18	3.02
Minimal	2 % glucose	4.90	4.67

^aCells harvested during stationary phase.

Slonimski (1957) supplemented with histidine (10 mg/mL). Depending on the type of experiment, other amino acids, tryptone, and purine and pyrimidine bases were supplied to the minimal medium.

Yeast growth. The strains were incubated at 30 °C with shaking at approximately 1.5 Hz and absorbance was read in a Spectronic-100 (Bausch and Lomb) spectrophotometer. They were harvested at mid-exponential phase.

Enzyme preparation and assay. Ten cells per nL were inoculated in 200 mL of the medium specified above and grown in conical flasks at 30 °C for 18 to 24 h with shaking. The cells were harvested by centrifugation, washed twice with deionized water and resuspended in 5 mM glycylglycine buffer (pH 7.6).

Cells were disrupted in a Braun cell homogenizer model MSK (Browill) for 80 s (50 s first velocity, 30 s second velocity). During disruption the flask was cooled by a stream of CO₂. The cell extract was immediately centrifuged (25 000 *g*, 20 min) in a MSE HS-18 centrifuge. The supernatant was desalted in a PD-10 Sephadex G-25 column previously equilibrated with buffer. Glucose-6-phosphate dehydrogenase activity was determined according to Kuby and Noltmann (1966) and 6-phospho-

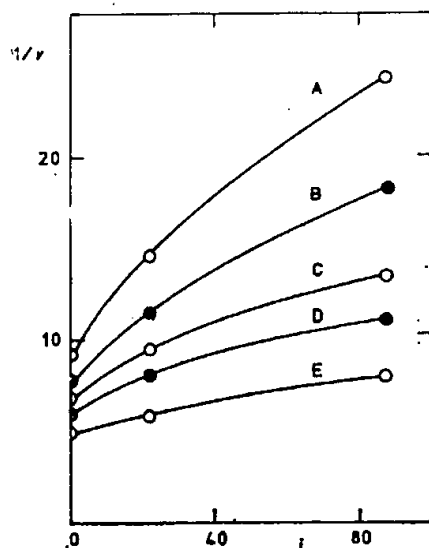


FIG. 1. Dixon plot (initial rate *v* expressed in ΔA_{240} per min) against concentration of NADPH (*i*) in μM . Curves A–E were obtained at the following concentrations of NADP as substrate (μM): 16.8, 33.5, 41.9, 67.1, and 167.7.

TABLE II. Effect of medium on glucose-6-phosphate dehydrogenase

Medium
Minimal
Complete

gluconate dehydrogenase following the method of Layne (1957). Reagents and bases were from Merck.

RESULTS

When yeast cells were grown in a rich medium and glucose-6-phosphate dehydrogenase activity was determined, it was found that the activity was higher than in rich medium.

TABLE III. Effect of amino acids on glucose-6-phosphate dehydrogenase (decarboxylating) activity

Amino acid
Tryp
Ala,
Gly,
Met,
Thr,
Glu,
Phe,
Ala,
His,
Ser
His,
His

hydrogenase (decarboxylating)

at/mg

PGD
2.25
2.57
2.58
3.02
4.67

depending on the type of pyrimidine bases were

ring at approximately 1 and 1.5 (Lomb) spectro-

culated in 200 mL of $^{\circ}\text{C}$ for 18 to 24 h with twice with deionized

SK (Browill) for 80 s. The flask was cooled by ice (25 000 g, 20 min) in a PD-10 Sephadex column. Glucose 6-phosphate dehydrogenase (1966) and 6-phospho-

initial rate v expressed in $\mu\text{mol NADPH}$ per concentration of NADPH were obtained at the following NADP as substrate (μM): 167.7.

TABLE II. Effect of nitrogenous compounds on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (decarboxylating) activities in a medium with 2 % glucose

Medium	Addition	nkat/mg	
		GPD	PGD
Minimal	none	5.55	5.40
	tryptone and tryptophan	3.12	2.50
	purines and pyrimidines	4.85	5.22
	tryptone, tryptophan, purines and pyrimidines	3.78	2.77
Complete	none	2.60	2.60

gluconate dehydrogenase was assayed according to Pontremoli and Grazi (1966) by following the rate of reduced NADPH production in a Unicam SP 1800 spectrophotometer with recorder at 340 nm. Protein was determined by the biuret method (Layne 1957) using bovine serum albumin as standard.

Reagents. Yeast extract and peptone were from Oxoid, D-glucose and amino acids and bases were from British Drug Houses; 6-phosphogluconate, glucose 6-phosphate (disodium salt) and MgCl_2 were from Koch-Light; NADP and glycylglycine were from Merck.

RESULTS

When yeast cells are grown in minimal medium containing glucose, the specific activities of GPD and PGD are 2.6 and 2.1 times higher, respectively, in relation to those in a rich medium (Table I). This agrees well with the values reported by Lagunas and Gancedo (1973) where the contribution of the oxidative hexose monophosphate pathway to glucose catabolism is 2.8 times higher in yeast grown in minimal medium than in rich medium.

TABLE III. Effect of amino acids on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (decarboxylating) from cells grown in minimal medium with 2 % glucose

Amino acid added	nkat/mg	
	GPD	PGD
Tryptone plus Trp	1.87	2.28
Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val	2.22	2.45
Glu, His, Ile, Lys, Phe, Pro, Thr, Val	3.30	3.32
Ala, Arg, Asp, Cys, Gly, His, Leu, Met, Trp, Tyr, Ser	2.70	2.80
His, Phe, Trp, Tyr	5.22	4.23
His	4.93	4.10

TABLE IV. Effect of various compounds on 6-phosphogluconate dehydrogenase (decarboxylating) activity

Compound added	Concentration mM	PGD activity nkat/mg
None	—	2.55
Fructose 1,6-bisphosphate	0.34	1.53
GTP	0.31	1.78
NaH ₂ PO ₄	0.21	1.98

Since the main components present in rich medium but absent from minimal medium are amino acids and purine and pyrimidine bases one might expect any one of these compounds to change the enzyme activities. Table II suggests that the amino acids in the medium included in the form of tryptone and tryptophan are responsible for these changes. Further confirmation is obtained from the results of Table III, since the presence in minimal medium of a mixture of 18 amino acids caused a similar effect on enzyme activities as growth in rich medium. However, when fewer amino acids were added to the minimal medium, variable values of enzyme activities were obtained.

Modulation of GPD and PGD activities by several physiological effectors and especially by the NADP⁺/NADPH ratio has been shown to be of primary importance in the regulation of the pentose phosphate dehydrogenases from different sources (Pontremoli and Grazi 1969; Tanaka *et al.* 1976; Dyson and D'Orazio 1971; Pearce and Rosemeyer 1974). These considerations led us to investigate whether any of these factors had an influence at the level of PDG activity in *S. cerevisiae*. We found NADPH to be a potent inhibitor of the enzyme, competing with NADP⁺ in a partial fashion (see the nonlinear Dixon plot in Fig. 1). While the K_m for NADP was 26 μ M, the K_i for NADPH read from a Lineweaver-Burk plot was 14 μ M. This value is to be compared with that of human erythrocyte PGD where a K_i of 30 μ M for NADPH was reported by Pearce and Rosemeyer (1974). However, considering the partial character of the inhibition we derived (see Kotyk and Horák 1977) the true K_i to be only 11.5 μ M.

Table IV shows that phosphate ions, GTP and fructose 1,6-bisphosphate also inhibited PGD activity.

DISCUSSION

There is good agreement that the pentose phosphate pathway provides most if not all of the NADPH that is needed by the cells of *S. cerevisiae* when grown on glucose (Lagunas and Gancedo 1973). NADPH, ribose 5-phosphate and erythrose 4-phosphate, known intermediates of the pentose phosphate pathway, are needed for the biosynthesis of several amino acids (Mahler and Cordes 1971). Therefore, for yeast growing in a medium supplemented with amino acids, a great decrease of cellular NADPH requirement can be expected and actually the activities of GPD and PGD decrease. The activities of these enzymes change by a factor similar to the one reported for the change in the rate of the pathway between cells grown in minimal medium and rich medium. The interrelations in the conversion of one amino acid to another as well as the complexity in the regulation of its metabolism which is not completely elucidated makes it difficult to attribute a single amino acid or even a group of amino acids which utilizes NADPH for its biosynthesis, the ability to change the activities of GPD and PGD.

The finding and phosphate control mechanism. Particularly, this compound in *Schizosaccharomyces cerevisiae* is a pyruvate kinase. This metabolic glycolytic and and Rosemeyer idea that not pentose phosphate

The authors are concerned with this

REFERENCES

- AFOLAYAN A.: *J. chem.* 11, 4172
- DIXON M.: *The*
- DYSON J.E.D.: *J. catalytic activ*
- EGGLESTON L.V.
- GALZY P., SLONI
- lc glucosidase
- GANCEDO J.M.,
- yeasts and its
- GANCEDO J.M.,
- in *Saccharomyces*
- (1973).
- HOLTEN D., *Pro*
- NADP/NADPH
- KOTYK A., HORÁK
- KUBY S.A., *Not*
- p, 116 in *W.A.*
- LAGUNAS R., *G.*
- cerevisiae*. *Euk*
- LAYNE E.: *Spec*
- S.P. Colowick
- MAHLER H.R., *G*
- PEARCE B.M.F.,
- cyto enzyme
- PONTREMOLI S.,
- Methods in En*
- PONTREMOLI S.,
- Comprehensive*
- SAPAG-HAGAR M.
- and glucose-6-
- SOLS A., SALAS X
- Academic Pre
- TANAKA A., *Tai*
- gluconate dehy
- 40, 1829 (206)

use (decarboxylating) activity

PGD activity nkat/mg
2.55
1.53
1.78
1.98

sent from minimal me-
might expect any one
e II suggests that the
ne and tryptophan are
ned from the results of
ture of 18 amino acids
ich medium. However,
um, variable values of

biological effectors and
of primary importance
from different sources
D'Orazio 1971; Pearse
te whether any of these
l. *cerevisiae*. We found
ith NADP⁺ in a partial
for NADP was 26 μ M,
14 μ M. This value is to
of 30 μ M for NADPH
considering the partial
& 1977) the true K_1 to

1,6-bisphosphate also

hway provides most if
evisiae when grown on
osphate and erythrose
pathway, are needed
s 1971). Therefore, for
s, a great decrease of
the activities of GPD
by a factor similar to
between cells grown in
onversion of one amino
ts metabolism which is
gle amino acid or even
nthesis, the ability to

The finding of the inhibition of PGD by NADPH, fructose 1,6-bisphosphate, GTP and phosphate could be of significance in the regulation of enzyme activity by a fine control mechanism whereby instant changes of PGD activity could take place. Particularly interesting is the inhibition caused by fructose 1,6-bisphosphate since this compound exerts a positive effect on two principal glycolytic enzymes of *Saccharomyces cerevisiae*: 6-phosphofructokinase (EC 2.7.1.11) (Sols and Salas 1969) and pyruvate kinase (Gancedo *et al.* 1967). Therefore, a change in the concentration of this metabolite could regulate the level of glucose 6-phosphate which enters the glycolytic and pentose phosphate pathways. A similar view was put forth by Pearse and Rosemeyer (1974) for human erythrocytes. The above evidence supports the idea that not only GPD but also PGD can play a role in regulating the rate of the pentose phosphate pathway in yeast.

The authors wish to express their thanks to Prof. A. Kotyk for his useful advice and critical comments concerning this work.

REFERENCES

- AFOLAYAN A.: Regulation and kinetics of glucose-6-phosphate dehydrogenase from *Candida utilis*. *Biochem.* 11, 4172 (1972).
- DIXON M.: The determination of enzyme inhibitor constants. *Biochem.J.* 55, 170 (1963).
- DYSON J.E.D., D'ORAZIO R.E.: 6-Phosphogluconate dehydrogenase from sheep liver: Inhibition of the catalytic activity by fructose 1,6-diphosphate. *Biochem.Biophys.Res Commun.* 43, 183 (1971).
- EGGLESTON L.V., KREBS H.A.: Regulation of the pentose phosphate cycle. *Biochem.J.* 138, 425 (1974).
- GALZY P., SLONIMSKI P.P.: Variations de la levure au cours de la croissance sur l'acide lactique ou sur le glucose comme seule source de carbone. *C.R.Acad.Sci. (Paris)* 245, 2423 (1957).
- GANCEDO J.M., GANCEDO C., SOLS A.: Regulation of the concentration or activity of pyruvate kinase in yeasts and its relationship to gluconeogenesis. *Biochem.J.* 102, 23c (1967).
- GANCEDO J.M., LAGUNAS R.: Contribution of the pentose phosphate pathway to glucose metabolism in *Saccharomyces cerevisiae*: A critical analysis on the use of labelled glucose. *Plant Sci.Lett.* 1, 193 (1973).
- HOLTEN D., PROCSAL D., CHANG H.L.: Regulation of pentose phosphate pathway dehydrogenases by NADP/NADPH ratios. *Biochem.Biophys.Res Commun.* 68, 436 (1976).
- KOTYK A., HORÁK J.: *Enzyme Kinetics*. (In Czech) Academia, Prague 1977.
- KUBY S.A., NOLTMANN E.A.: Glucose-6-phosphate dehydrogenase (crystalline) from brewer's yeast, p. 116 in W.A. Wood (Ed.): *Methods in Enzymology*, Vol. IX. Academic Press, New York 1966.
- LAGUNAS R., GANCEDO J.M.: Reduced pyridine nucleotide balance in glucose growing *Saccharomyces cerevisiae*. *Eur.J.Biochem.* 37, 90 (1973).
- LAYNE E.: Spectrophotometric methods and turbidimetric methods for measuring proteins, p. 447 in S.P. Colowick, N.O. Kaplan ((Eds.): *Methods in Enzymology*, Vol. III. Academic Press, New York 1957.
- MAHLER H.R., CORDES E.H.: *Biological Chemistry*. Harper & Row, New York 1971.
- PEARSE B.M.F., ROSEMEYER M.A.: Human 6-phosphogluconate dehydrogenase. Purification of the erythrocyte enzyme and the influence of ions and NADPH on its activity. *Eur.J.Biochem.* 42, 213 (1974).
- PONTREMOLI S., GRAZI E.: Phosphogluconate dehydrogenase. Crystalline, p. 137 in W.A. Wood (Ed.): *Methods in Enzymology*, Vol. IX. Academic Press, New York 1966.
- PONTREMOLI S., GRAZI E.: Hexose-monophosphate oxidation, p. 163 in M. Florkin, E.H. Stotz (Eds.): *Comprehensive Biochemistry*, Vol. XVII. Elsevier, New York 1969.
- SAPAG-HAGAR M., LAGUNAS R., SOLS A.: Apparent unbalance between the activities of 6-phosphogluconate and glucose-6-phosphate dehydrogenase in rat liver. *Biochem.Biophys. Res Commun.* 50, 179 (1973).
- SOLS A., SALAS M.L.: Phosphofructokinase, p. 436 in W.A. Wood (Ed.): *Methods in Enzymology*, Vol. IX. Academic Press, New York 1966.
- TANAKA A., TAKENAKA Y., HIRAI M., FUKUI S.: Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of *Candida tropicalis*. Partial purification and some properties. *Agr.Biol.Chem.* 40, 1829 (206).

STIC-ILL

From: Steadman, David (AU1652)
Sent: Monday, March 19, 2001 4:35 PM
To: STIC-ILL
Subject: Literature/ILL Request

Mic
QP501. F4
Adms

Art Unit: 1652
Room #: 8B-11
Serial #:09/621,448

Please provide the following references:

- 1) FEBS Lett 1996 May 27;387(1):7-10
Reoxidation of the NADPH produced by the pentose phosphate pathway is necessary for the utilization of glucose by Kluyveromyces lactis rag2 mutants.
Gonzalez Siso MI, Freire Picos MA, Cerdan ME
- 2) Microbiology 1995 Feb;141 (Pt 2):385-91
In Saccharomyces cerevisiae deletion of phosphoglucose isomerase can be suppressed by increased activities of enzymes of the hexose monophosphate pathway.
Dickinson JR, Sobanski MA, Hewlins MJ
- 3) J Bacteriol 1997 Apr;179(8):2724-30
Interruption of the phosphoglucose isomerase gene results in glucose auxotrophy in Mycobacterium smegmatis.
Tuckman D, Donnelly RJ, Zhao FX, Jacobs WR, Connell ND
- 4) Eur J Biochem 1993 Oct 1;217(1):469-77
The role of the NAD-dependent glutamate dehydrogenase in restoring growth on glucose of a Saccharomyces cerevisiae phosphoglucose isomerase mutant.
Boles E, Lehnert W, Zimmermann FK
- 5) Appl Environ Microbiol 1991 Oct;57(10):2995-9
Deletion of *pgi* alters tryptophan biosynthesis in a genetically engineered strain of Escherichia coli.
Mascarenhas D, Ashworth DJ, Chen CS

Thank you,
David Steadman